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TRANSCRIPTIONAL CONTROL OF LIVER METABOLISM AND DISEASE BY NUCLEAR RECEPTOR-COREPRESSOR NETWORKS

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Transcriptional control of liver metabolism and disease by nuclear receptor-corepressor networks

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Liver lipid metabolism is coordinated via transcriptional networks composed of transcription factors and coregulators. Disturbance of such networks leads to metabolic dysregulation and is linked to the progression of obesity-related metabolic disorders, such as non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease. Lipid-sensing nuclear receptors, particularly liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs), play a crucial role in cholesterol and triglyceride regulation and have emerged as significant targets for drug development. The major obstacles of targeting nuclear receptors are the undesired and often unpredictable side effects due to their genome-wide activities in multiple cell-types. Therefore, investigating the associated coregulators and the post-translational modifications might help to better understand the gene-, cell-type- and signal-specific regulation of nuclear receptors, especially upon pathophysiological conditions. Of particular interest is G-protein pathway suppressor 2 (GPS2), subunit of a fundamental corepressor complex, which seems involved in cholesterol homeostasis and anti-inflammatory crosstalk in a variety of tissues. Although lipid dysregulation and inflammation are two major mechanisms to promote metabolic disorders, the role of GPS2 in the development of those diseases remained enigmatic. The objective of this thesis was therefore to characterize the roles and relationship of GPS2, along with the corepressor complex, to individual transcription factors/nuclear receptors at the physiological and genomic level with an emphasis on obesity-associated metabolic disorders.

In **Paper I**, we discovered a hitherto unknown function of GPS2 in the progression of NAFLD to non-alcoholic steatohepatitis (NASH). We demonstrated that GPS2 selectively repressed PPARα activity in liver lipid catabolism via the corepressor complex. Hepatocyte-specific *Gps2* knockout mice were protected from diet-induced liver steatosis and fibrosis, by enhancing fatty acid oxidation and ketogenesis as result of PPARα de-repression and epigenome alterations. Further, by studying human NAFLD/NASH biopsies we found that *GPS2* expression positively correlated with fibrogenic and inflammatory gene expression. Thus, the selective modulation of GPS2-PPARα interactions could be of therapeutic interest for NAFLD/NASH.

In **Paper II,** we identified GPS2 as a pivotal regulator of lipopolysaccharides (LPS)-induced *ABCA1* expression and cholesterol efflux, independent of LXR and the corepressor complex, in inflammatory macrophages. This study advanced our understanding of GPS2 in linking obesity-associated inflammation to cardiovascular diseases. As GPS2 is downregulated

whilst the circulating endotoxin is elevated in obesity, the LPS-GPS2-ABCA1 axis may provide a potential link to explain the increased cardiovascular risk in obesity and T2D.

In **Paper III,** we demonstrated that LXRα phosphorylation played a crucial role in NAFLD progression in mice. In phosphorylation-deficient LXRα knockin mice, diet-induced NAFLD was attenuated by repressing the expression of multiple inflammatory and fibrotic mediators. We uncovered a unique group of diet-specific phosphorylation-sensitive LXRα target genes in liver, different from those affected by loss of LXRα. Moreover, evidence is provided that phosphorylation may modulate the interaction of LXRα with the corepressor complex. This study highlights the role of post-translational modifications in defining the gene-selective transcriptional activity of nuclear receptors.

In conclusion, this thesis revealed novel insights into the multifaceted regulatory roles of nuclear receptors and GPS2 in altering transcriptional and epigenomic networks linked to metabolic and inflammatory processes. These insights may contribute to the better understanding of the development of obesity-associated metabolic disorders and to novel intervention strategies.

LIST OF SCIENTIFIC PAPERS

I. **Ning Liang**, Anastasius Damdimopoulos, Saioa Goñi, Zhiqiang Huang, Lise-Lotte Vedin, Tomas Jakobsson, Marco Giudici, Osman Ahmed, Matteo Pedrelli, Serena Barilla, Fawaz Alzaid, Arturo Mendoza, Tarja Schröder, Raoul Kuiper, Paolo Parini, Anthony Hollenberg, Philippe Lefebvre, Sven Francque, Luc Van Gaal, Bart Staels, Nicolas Venteclef, Eckardt Treuter & Rongrong Fan (2019). Hepatocyte-specific loss of GPS2 in mice reduces non-alcoholic steatohepatitis via activation of PPARα. Nature communications 10, 1684. doi: 10.1038/s41467-019-09524-z.

II. Zhiqiang Huang, **Ning Liang**, Anastasius Damdimopoulos, Rongrong Fan & Eckardt Treuter (2019). G protein pathway suppressor 2 (GPS2) links inflammation and cholesterol efflux by controlling lipopolysaccharide-induced ATP-binding cassette transporter A1 expression in macrophages. FASEB J 33, 1631-1643. doi: 10.1096/fj.201801123R.

III. Natalia Becares, Matthew C. Gage, Maud Voisin, Elina Shrestha, Lucia Martin-Gutierrez, **Ning Liang**, Rikah Louie, Benoit Pourcet, Oscar M. Pello, Tu Vinh Luong, Saioa Goñi, Cesar Pichardo-Almarza, Hanne Røberg-Larsen, Vanessa Diaz-Zuccarini, Knut R. Steffensen, Alastair O'Brien, Michael J. Garabedian, Krista Rombouts, Eckardt Treuter & Inés Pineda-Torra (2019). Impaired LXRα phosphorylation attenuates progression of fatty liver disease. Cell reports 26, 984-995 e986. doi: 10.1016/j.celrep.2018.12.094.

Related publications not included in this thesis

Rongrong Fan, Amine Toubal, Saioa Goñi, Karima Drareni, Zhiqiang Huang, Fawaz Alzaid, Raphaelle Ballaire, Patricia Ancel, **Ning Liang**, Anastasios Damdimopoulos, Isabelle Hainault, Antoine Soprani, Judith Aron-Wisnewsky, Fabienne Foufelle, Toby Lawrence, Jean-Francois Gautier, Nicolas Venteclef & Eckardt Treuter (2016). Loss of the co-repressor GPS2 sensitizes macrophage activation upon metabolic stress induced by obesity and type 2 diabetes. Nature Medicine 22, 780-791. doi: 10.1038/nm.4114.

Ning Liang, Rongrong Fan, Saioa Goñi & Eckardt Treuter (2019). Preparation of frozen liver tissues for integrated omics analysis. Methods in Molecular Biology 1951, 167-178. doi: 10.1007/978-1-4939-9130-3_13.

Karima Drareni, Raphaëlle Ballaire, Serena Barilla, Mano J. Mathew, Amine Toubal, Rongrong Fan, **Ning Liang**, Catherine Chollet, Zhiqiang Huang, Maria Kondili, Fabienne Foufelle, Antoine Soprani, Ronan Roussel, Jean-François Gautier, Fawaz Alzaid, Eckardt Treuter & Nicolas Venteclef (2018). GPS2 deficiency triggers maladaptive white adipose tissue expansion in obesity via HIF1A activation. Cell reports 24, 2957-2971 e2956. doi: 10.1016/j.celrep.2018.08.032

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid residues
ABC	ATP binding cassette
ABCA1	ATP binding cassette subfamily A member 1
ABCG1	ATP-binding cassette subfamily G member 1
ABCG5	ATP-binding cassette subfamily G member 5
ABCG8	ATP-binding cassette subfamily G member 8
Ac	Acetylation
ACC	Acetyl-CoA carboxylase
Alb	Albumin
Akt	Protein kinase B
Apoe	Apolipoprotein E
APR	Acute phase response
BA	Bile acid
C/EBP	CCAAT-enhancer-binding proteins
CCL2	C-C motif chemokine ligand 2, alias MCP-1 (monocyte chemoattractant protein 1)
CCL7	C-C motif chemokine ligand 7
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation coupled with next-generation sequencing
ChREBP	carbohydrate response element-binding protein
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1
Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1
Cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10
Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14
Cyp4a32	Cytochrome P450, family 4, subfamily a, polypeptide 32
DAD	Deacetylase-activation domain
DKO	Double knockout
DNL	*de novo* lipogenesis
ERRα	Estrogen-related receptor alpha, nuclear receptor subfamily 3, group B, member 1
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FFA	Free fatty acid
FGF	Fibroblast growth factors
FOXA2	Forkhead box protein A2, hepatocyte nuclear factor 3-beta
FXR	Farnesoid X receptor, nuclear receptor subfamily 1, group H, member 4
G9a	Euchromatic histone-lysine N-methyltransferase 2
GPS2	G-protein pathway suppressor 2
HAT	Histone acetyltransferase
HCC	Hepatocellular carcinoma
HDAC3	Histone deacetylase 3
HDL	High-density lipoprotein
HFD	High-fat diet

HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase
HNF4α	Hepatocyte nuclear factor 4 alpha, nuclear receptor subfamily 2, group A, member 1
HNF6	Hepatocyte nuclear factor 6
IL-12β	Interleukin 12 beta
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
KDM	Lysine (K)-specific demethylase
KO	Knockout
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LKO	Liver/hepatocyte-specific knockout
LPS	Lipopolysaccharides
LRH1	Liver receptor homolog 1, nuclear receptor subfamily 5, group A, member 2
LRP	LDL receptor–related protein
LXRα	Liver X receptor alpha, nuclear receptor subfamily 1 group H member 3
LXRβ	Liver X receptor beta, nuclear receptor subfamily 1 group H member 2
MCD	Methionine- and choline- deficient diet
Me	Methylation
MKO	Macrophage-specific knockout
mTOR	Mammalian target of rapamycin
N-CoEx	NR corepressor-coactivator-exchange factors
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCOR	Nuclear receptor corepressor, alias N-CoR, NCOR1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NR	Nuclear receptor
PDK4	Pyruvate dehydrogenase lipoamide kinase isozyme 4
PPARα	Peroxisome proliferator-activated receptor alpha, nuclear receptor subfamily 1, group C, member 1
PPARγ	Peroxisome proliferator-activated receptor gamma, nuclear receptor subfamily 1, group C, member 3
PPARδ	Peroxisome proliferator-activated receptor delta, nuclear receptor subfamily 1, group C, member 2
PROX1	Prospero homeobox protein 1
PTM	Post-translational modification
RAR	Retinoic acid receptors, nuclear receptor subfamily 1, group B, members 1,2,3
RCT	Reverse cholesterol transport
RD	Repression domains
RevErbα	Nuclear receptor subfamily 1, group D, member 1
RevErbβ	Nuclear receptor subfamily 1, group D, member 2
RIP140	Receptor-interacting protein 140, alias NRIP1 (nuclear receptor-interacting protein 1)
RORE	Retinoic acid receptor-related orphan receptor binding element
RORα	Retinoic acid receptor-related orphan receptor, nuclear receptor subfamily 1 group F member 1

RXR	Retinoid X receptors, nuclear receptor subfamily 2, group B, members 1,2,3
S6K2	S6 kinase 2
SCD1	Stearoyl-CoA desaturase-1
SHP	Small heterodimer partner, nuclear receptor subfamily 0, group B, member 2
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor, alias NCOR2
SREBP-1c	Sterol regulatory element binding-protein 1c
SUMO	Small ubiquitin-like modifier
T2D	Type 2 diabetes
TBL1	Transducin β-like protein 1
TBLR1	Transducin β-like protein-related 1
TF	Transcription factor
TG	Triglyceride
TNFα	Tumor necrosis factor alpha
TR	Thyroid hormone receptors, nuclear receptor subfamily 1, group A, members 1,2
VLDL	Very-low-density lipoprotein
WT	Wild type

1 INTRODUCTION

1.1 Non-alcoholic fatty liver disease (NAFLD)

The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing globally with obesity (1). NAFLD is a chronic liver metabolic disorder which affects up to 30% of the world's adult population currently (2-4). NAFLD is characterized by abnormal liver lipid accumulation and ranges from simple hepatic steatosis to severe non-alcoholic steatohepatitis (NASH), depending on the extent of steatosis, lobular inflammation and ballooning degeneration of the hepatocytes (5, 6). NASH is typically accompanied with peri-cellular fibrosis in liver histology, and may potentially progress to more advanced liver diseases, such as cirrhosis, liver failure and hepatocellular carcinoma (HCC) (7, 8). The irreversible transition to cirrhosis and HCC is the primary cause of mortality associated with NASH (7). NAFLD is the second leading cause for liver transplantation in the USA and is predicted to become the leading one in the coming years (9).

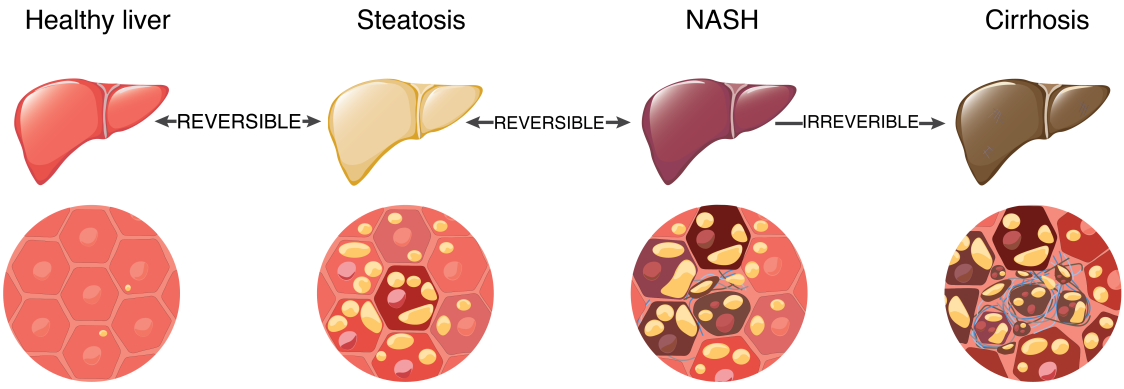

Figure 1 The Spectrum of NAFLD. NAFLD encompasses a spectrum of disease, from left to right: healthy liver; steatosis, defined by more than 5% hepatocytes with fat accumulation; NASH, which is characterized by steatosis, inflammation, and hepatocyte cell ballooning associated with or without liver fibrosis; Cirrhosis, with its characteristic collagen bands surrounding liver nodules.

Presence of metabolic syndromes, including abnormal obesity, insulin resistance and hyperglycemia, atherogenic dyslipidemia, pro-inflammation and systemic hypertension (10), has the most explicit biologic link to the progression of NAFLD (8). This association is bidirectional, meaning that both metabolic syndrome and NAFLD contribute to the risk for each other in a vicious circle (7, 11, 12). Among these metabolic syndromes, T2D is the most

potent risk factor for the NASH progression in NAFLD patients (8, 13, 14). More than 70% of individuals with T2D have NAFLD (13, 14), among which, the prevalence of NASH as well as advanced fibrosis is remarkably enriched compared to nondiabetics with NAFLD (15). In addition, metabolic syndrome also promotes the development of adverse cardiovascular diseases and increases the overall mortality in NAFLD patients (16, 17).

Insulin resistance has been characterized as the crucial pathophysiological factor in NAFLD for many years (18). All forms of NAFLD are strongly correlated with both hepatic and peripheral insulin resistance (19, 20), and NAFLD progression further worsens insulin sensitivity (21). However, more and more evidence showed that NASH could occur in the absence of insulin resistance (22). This suggested that hepatic steatosis may begin as a simple imbalance of liver lipid metabolism, in which the formation of free fatty acids (FFAs) exceed their utilization. When excessive FFAs are supplied or their disposal is impaired, their metabolites may serve as substrates for the generation of lipotoxic species, and induce hepatocellular stress, hepatocyte injury and death, leading to fibrogenesis and genomic instability (8).

The progression rate of NAFLD is highly variable among individuals and has different clinical manifestations (8), which reflects the diverse but convergent impact of the environment, the microbiome, metabolism, comorbidities and genetic risk factors (23, 24). Thus, clarifying the contributions of each factor and sub-classifying the disease based on individualized drivers could help to predict the disease progression accurately and apply more effective treatments. Multiple other factors such as insulin resistance, lipotoxicity, inflammation and genetic susceptibility act in parallel to trigger the disease development (2, 5, 6, 25).

Gene expression (transcriptome) analysis during NAFLD progression (26, 27) has identified a variety of differentially expressed marker genes, but it is still challenging to demonstrate whether the differential expression is also the cause of disease progression in humans.

Currently, there are no effective therapeutic strategies for NASH patients, which is largely due to our limited understanding of the underlying molecular events that control the disease initiation and development (5, 6). Thus, major efforts in the field are dedicated to identify potential targets to improve liver functions, for example, key factors which promote or prevent the progression of NAFLD.

1.2 Nuclear receptors in NAFLD

1.2.1 Imbalanced lipid metabolism

The liver is the major organ of glucose and lipid metabolism (28) and is composed of multiple cell types, mainly hepatocytes and immune cells. Its metabolic functions are tightly regulated by hormones and nuclear receptors (NRs).

Hepatic steatosis is the hallmark feature of NAFLD, which is caused by the imbalances of the processes that maintain liver energy homeostasis (29). Conceptually, excess triglyceride (TG) storage in liver may result from increased lipid acquisition (uptake of FFAs released by adipose tissue from the blood, uptake of TG from chylomicron remnant, and *de novo* lipogenesis (DNL)) and/or decreased lipid disposal (e.g., fatty acid oxidation (FAO) and very-low-density lipoprotein (VLDL) secretion) (Figure 2).

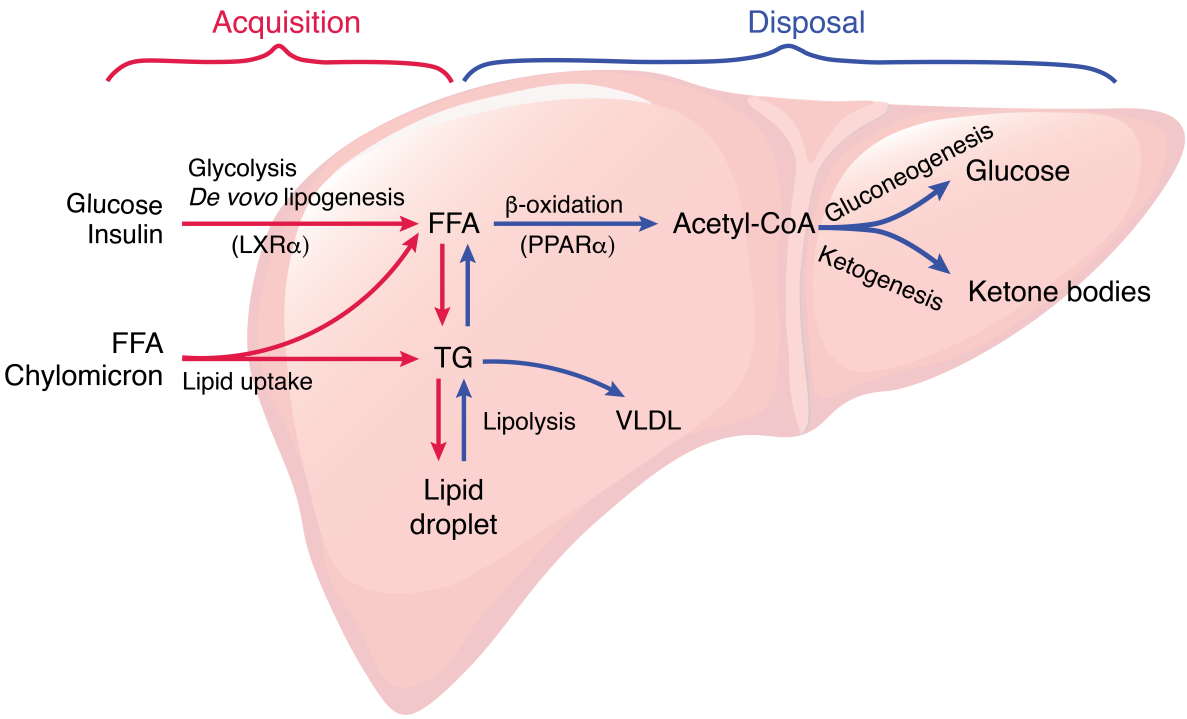

Figure 2 Liver lipid homeostasis. Highlighted are the processes involved in maintenance of lipid homeostasis. The lipid formation processes are colored red and lipid utilization processes are colored blue.

1.2.1.1 Lipid acquisition processes

Dietary TG in the remnant chylomicrons are delivered to the liver (30), and are transported into hepatocytes via the low-density lipoprotein (LDL) receptor (LDLR) and LDL receptor–related protein (LRP) (31-33).

The FFA released from adipose tissue due to adipose insulin resistance is the key contributor to excessive storage of hepatic TG (34). In fasted individuals with obesity and NAFLD, circulating FFA and DNL account for ~59% and ~26% of hepatic total TG respectively (35).

The liver is a major site of DNL, which is tightly governed by insulin and glucose status. Insulin and glucose activate two key transcription factors (TFs), sterol regulatory element binding protein-1c (SREBP-1c) (36) and carbohydrate response element binding protein (ChREBP) (37)), respectively. They both play critical roles in controlling the expression of several lipogenic genes, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN) and stearoyl-CoA desaturase-1 (SCD1) (38, 39). The insulin-induced SREBP-1c expression requires the participation of liver X receptor (LXR), a NR that also plays a key role in maintaining cellular cholesterol homeostasis (37, 40).

1.2.1.2 Lipid disposal processes

Liver also has a major role in distributing lipids to other organs (29). FFAs generated from DNL, along with those taken up from circulation, can further be esterified and store in lipid droplets as TG or exported from the liver as VLDL particles (41).

Another important aspect of lipid disposal is via FAO, which is tightly governed by peroxisome proliferator-activated receptor alpha (PPARα). Short-chain and medium-chain FFA utilization is mainly accomplished via the mitochondrial and the peroxisomal β-oxidation pathways. The very long-chain FFA (more than 20 carbons) are converted to dicarboxylic acid in the microsomal via ω-oxidation pathways before oxidization in the mitochondria. During this process, the introduction of a hydroxyl group onto the ω carbon, the most distant carbon from the carboxyl group of the FFA, is catalyzed by Cytochrome P450 omega hydroxylase, including Cyp4a10, Cyp4a14 and Cyp4a32. The acetyl-CoA, derived from β-oxidation, can then be converted to ketone bodies to be used as an energy source by other tissues. This process is termed as ketogenesis. The mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) is the key enzyme (42).

In line with this, in the past decades, many glucose- and lipid-sensing TFs/NRs have been discovered and validated to be involved in the progression of NAFLD. These key factors include, but are not limited to, fatty acid-sensing PPARs (43), oxysterol-sensing LXRs (44), bile acid (BA)-sensing farnesoid X receptors (FXRs) (45), and thyroid hormone receptor (TR) β (46).

Further targets are the key enzymes and hormones modulating the liver lipid and glucose metabolism, such as SREBP-1c, ChREBP (5), FASN (47), ACC (48), and fibroblast growth factors (FGFs) (49, 50), etc.

1.2.2 Mouse models

1.2.2.1 High-fat diet (HFD)-induced mouse liver steatosis model

The association between NAFLD and obesity led to the usage of an HFD. Besides the increased body weight, the HFD mice also shows hepatic steatosis, as evidenced by the increased haptic TG levels, hepatocyte ballooning and higher fasting serum glucose levels, suggesting hyperglycaemia and insulin resistance (51). HFD fed mice better mimic both the histopathology and pathogenesis of human NAFLD, as they exhibit the hallmark features observed in human NAFLD patients, including obesity and insulin resistance (52).

1.2.2.2 Methionine- and choline- deficient diet (MCD)- induced mouse liver fibrosis model

The MCD-induced mouse liver fibrosis model is one of the best characterized models for NAFLD studies. The lack of choline and methionine leads to hepatic TG accumulation, liver cell death, oxidative stress, distinct inflammation and early development of fibrosis (52). This model partially mimics the pathological changes of severe human NAFLD, including severe lipid accumulation, macrophage infiltration, liver fibrosis and hepatocyte apoptosis (52).

1.2.3 Nuclear receptors in lipid metabolism

1.2.3.1.1 Roles of PPARα in hepatic lipid metabolism

PPARs consist of three subtypes designated PPARα, PPARβ/δ, and PPARγ. The first member PPARα was identified in 1990 and named for its ability to induce peroxisome proliferation (53). PPARs ligands include different types of endogenous FFAs and their metabolites, and synthetic drugs, including fibrates and thiazolidinedione (54). Activated PPARs forms heterodimers with retinoid X receptors (RXRs), and bind to sequence-specific target elements, known as the PPAR response element, at the promoter region of various

target genes, regulating their transcription. All three PPAR subtypes are highly expressed in metabolically active tissues, such as the liver, adipose tissue, skeletal muscle, but they show different tissue distribution patterns and ligand specificities, highlighting their tissue specificity (55).

The PPARα functions have been well-characterized in the liver, where it controls fatty acid transport, microsomal fatty acid ω-oxidation, mitochondrial and peroxisomal fatty acid β-oxidation, and ketogenesis in response to feeding and fasting (55). Moreover, PPARα activation also ameliorates the detoxification of hydrogen peroxide to protect hepatocytes from oxidative stress, which has a crucial role during liver injury in NASH (5). In line with this, *Pparα* knockout (KO) in mice promotes HFD-induced hepatic TG accumulation, macrophage infiltration, oxidative stress and hepatocyte apoptosis (Abdelmegeed et al., 2011).

In addition, the ability of PPARα to alleviate NAFLD/NASH symptoms at different stages has been studied in other mouse models (43). MCD-fed *Pparα* KO mice developed more severe steatohepatitis than wild type (WT) mice (56). Administration of Wy-14643, a PPARα agonist, ameliorated MCD-induced liver fibrosis and steatohepatitis in WT mice, but had no effect in *Pparα* KO mice (56). In addition, Bezafibrate (pan-PPAR agonist) and GW501516 (PPARδ agonist) fed mice were protected from the MCD-induced hepatic TG accumulation, liver inflammation and hepatic stellate cells activation (57). On one hand, these agonists upregulated a variety of lipid catabolism genes to prevent intrahepatic lipid accumulation (56, 57). On the other hand, these agonists inhibited liver inflammation and fibrosis, as evidenced by the decreased inflammatory cytokines and chemokines expression, decreased fibrogenic genes expression and reduced numbers of activated hepatic stellate cells and macrophages (56-58). In addition, PPARα could also suppress the acute phase inflammatory response (59, 60).

Because PPARα is also highly expressed in adipose tissue, skeletal muscle in addition to liver, the hepatic role of PPARα was uncovered until the phenotyping of the recently generated liver-specific *Pparα* KO (LKO) mice. PPARα LKO impaired liver and whole-body fatty acid catabolism, resulting in hepatic TG accumulation during fasting, MCD and HFD feeding as well as aging. Evidence showed that hepatic PPARα responded to acute and chronic adipose tissue lipolysis, and was responsible for circadian FGF21 and fasting-induced FGF21 expression. Prolonged fasting caused hypoglycaemia and hypothermia in *Pparα* LKO mice, due to defective FAO and gluconeogenesis. Although *Ppara* LKO mice did not show age-

related increase in body weight as *Pparα* KO mice, they developed NAFLD and hypercholesterolaemia (61). All these findings underscore hepatocyte PPARα as a potential therapeutic target for NAFLD.

In addition to these preclinical (56, 62-64) studies, the clinical (65) studies further highlighted the fundamental impact of PPARα on NAFLD/NASH. In human liver biopsies, the *PPARα* expression negatively correlated with NASH presence and severity (e.g., steatosis, ballooning, NASH activity score and fibrosis), visceral adiposity and insulin resistance and positively correlated with adiponectin (65). In addition, the histological improvement was associated with increased expression of PPARa and its target genes (65). These data indicate that PPARα is a critical therapeutic target in NASH.

1.2.3.2 LXRs in fatty acid and cholesterol metabolism

As sterol-activated NRs, LXR ligands include oxysterols and certain intermediates in the cholesterol biosynthetic pathway (66-69). Once activated by ligands, LXRs heterodimerize with RXR to control cholesterol and lipid homeostasis by regulating the expression of multiple enzymes, transporters, and modulators involved in these processes (70).

Of the two subtypes, LXRα is highly expressed in metabolically active tissues and cell types such as the liver, adipose tissues and macrophages (66, 67). Thus, LXRα is mainly responsible for the activation of DNL genes, such as FASN, SCD1 and SREBP-1c, and contributes to liver steatosis (71). Consistently LXRα- and LXRβ-depleted *ob/ob* mice showed reduced hepatic steatosis and improved insulin sensitivity (72). Therefore, the lipogenic actions of LXRs could be a major obstacle in the development of LXR agonists as drugs for cardiovascular disease (70).

In addition, LXRs play a central role in cholesterol metabolism, including BA synthesis, cholesterol efflux and reverse cholesterol transport (RCT). In both *apolipoprotein E* (*Apoe)*- and *Ldlr*- KO mice, the synthetic LXR agonist inhibited the development of aortic lesions (73, 74). The cytochrome P450 7A1 (*Cyp7a1*), a gene encoding the rate-limiting enzyme in BA synthesis, was the first direct LXR target gene (67). Several members of the ATP binding cassette (ABC) family of membrane transporters, such as ABCA1, ABCG1, ABCG5 and ABCG8, are also direct LXR targets, which mobilize cholesterol from the peripheral tissues, enhance high-density lipoprotein (HDL) formation, and attenuate atherosclerosis (75-78).

Moreover, LXRs modulate inflammatory and immune pathways (79) and show anti-inflammatory and anti-fibrotic activities in experimental models of acute liver disease (80,

81). The role of LXRβ in trans-repression of the hepatic acute phase response (APR) will be discussed in the next section (82).

Notably, the expression of both LXRs and their target genes were found to be upregulated and positively correlated with intrahepatic inflammation and fibrosis in NAFLD patients (83, 84). In sum, all these studies suggest that LXRs are important therapeutic targets for liver and cardiovascular diseases. More efforts should be dedicated to dissect their beneficial and detrimental functions.

1.3 THE COREPRESSOR COMPLEX

The major obstacles of NR-targeting drugs are the undesired side effects associated with the genome-wide role of TFs in positively and negatively regulating transcription in a highly context-dependent manner. Therefore, it is necessary to further dissect and better understand the mechanisms of gene-, cell type- and signal-specific TF action to maintain the beneficial therapeutic outcomes of TF-targeting drugs while eliminating their side effects.

TF-interacting coregulators play critical roles in determining the TF activities and the associated chromatin states at specific gene loci. Coregulators, commonly categorized into corepressors and coactivators based on their positive or negative regulation of gene expression, often function highly context-specific (85). Extrahepatic signals trigger the exchange of corepressor and coactivator complexes at the TF-containing regulatory chromatin elements, mainly promoters and enhancers, to regulate the transcription process. This kind of regulation is highly cell-type- or gene-specific, and in some cases the change of the sensitivity of cellular responses linked with the progression of human diseases. Most coregulators cooperate within histone-modifying enzyme complexes to change local chromatin environment, including adding or removing histone methylation and acetylation, which is further associated with gene expression or repression. The histone modification status, termed as epigenome, can be “read” by chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq). In addition, ChIP-seq has been applied to identify the genome-wide chromatin binding of TFs and coregulators, termed as cistrome (Figure 3). However, due to the lack of high-quality antibodies combined with the fact that coregulators unlike TFs do not bind chromatin directly, the application of ChIP-seq to coregulators is still limited. As a result, how the coregulator/TF switch is coordinated by extracellular signals such as hormones and inflammatory cytokines, and how cell- and gene-selectivity of the coregulator regulation is conducted through TF-dependent and -independent mechanisms, remains currently poorly understood. In terms of liver physiology and disease, such

understanding is extremely important since hepatocytes are constantly exposed to chemicals, nutrients and hormones (85, 86).

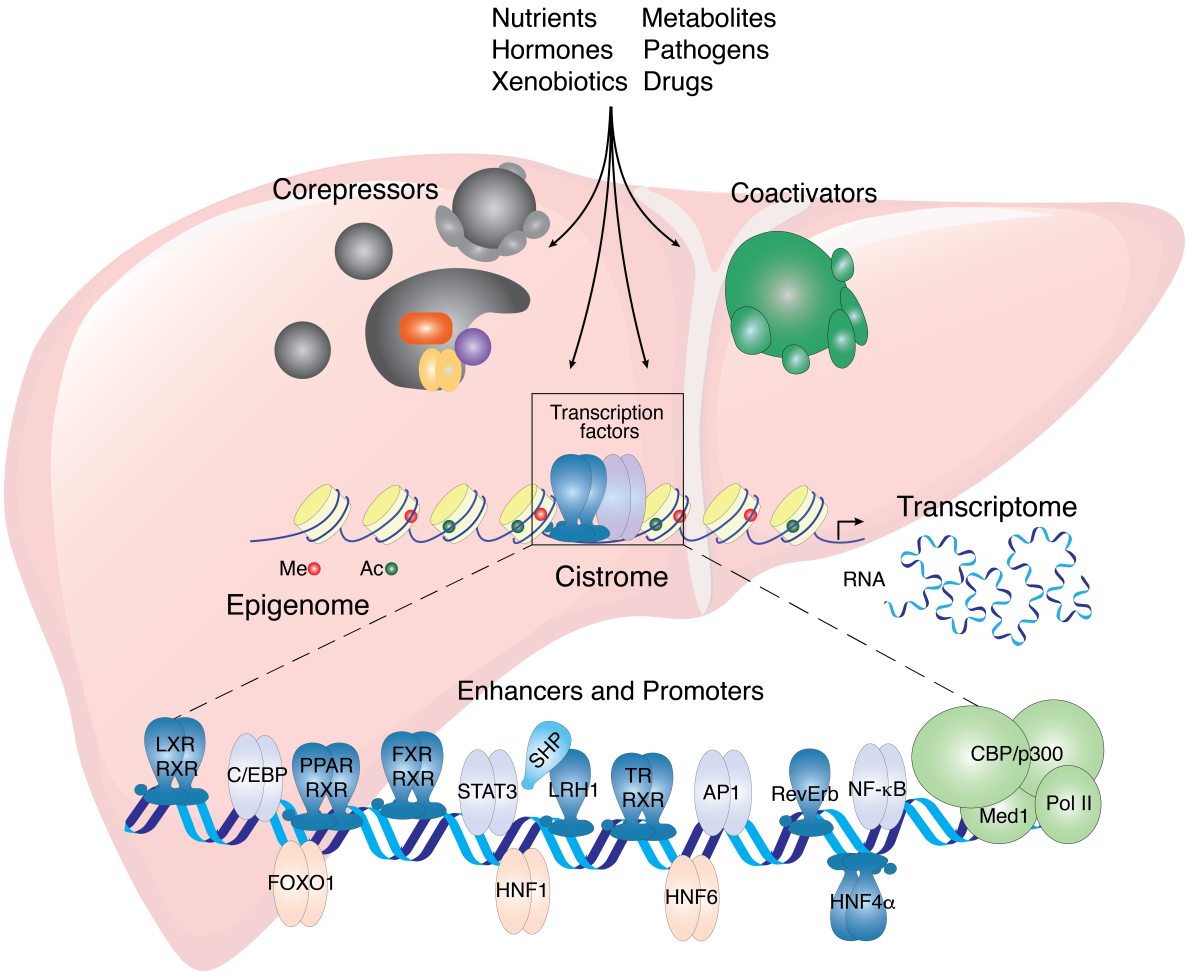

Figure 3 Components of regulatory transcriptional networks in hepatocytes. Highlighted are the key components of hepatocyte regulatory transcriptional networks. Both TFs and coregulators (including corepressors or coactivators) are responsible to transform multiple extracellular signals into physiological pathways and transcriptome changes. To fulfill this, coregulators are recruited to cis-regulatory chromatin elements, enhancers and promoters, by different classes of TFs. These TFs include metabolic NRs (*in dark blue*), inflammatory TFs (*in light blue*) and hepatocyte lineage-determining TFs (*in light orange*). Coregulators function within multi-protein histone-modifying enzyme complexes to change histone modifications, including (de-)acetylation (Ac) and (de-)methylation (Me), associated with the silencing or activation of gene expression. ChIP-seq has been applied to identify hepatocyte cistromes, i.e. the genome-wide binding sites of TFs and coregulators, as well as epigenomes, i.e. the genome-wide chromatin modifications such as active and repressive histone marks. Ac (acetylation), Me (methylation).

During the past decade, one particular corepressor complex has become the probably most-studied and best-understood physiological relevant coregulator in the liver. This multiprotein

complex contains the core subunits histone deacetylase 3 (HDAC3), nuclear receptor corepressor (NCOR, also known as NCOR1), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT, also known as NCOR2). Additional core subunits are G-protein pathway suppressor 2 (GPS2), transducin β-like protein 1 (TBL1, also known as TBL1X) and TBL-related 1 (TBLR1, also known as TBL1XR1) (86-94). A variety of LKO mice, and RNAi-mediated knockdown models, have been generated for most of the subunits. The major phenotypes are summarized in Table 1.

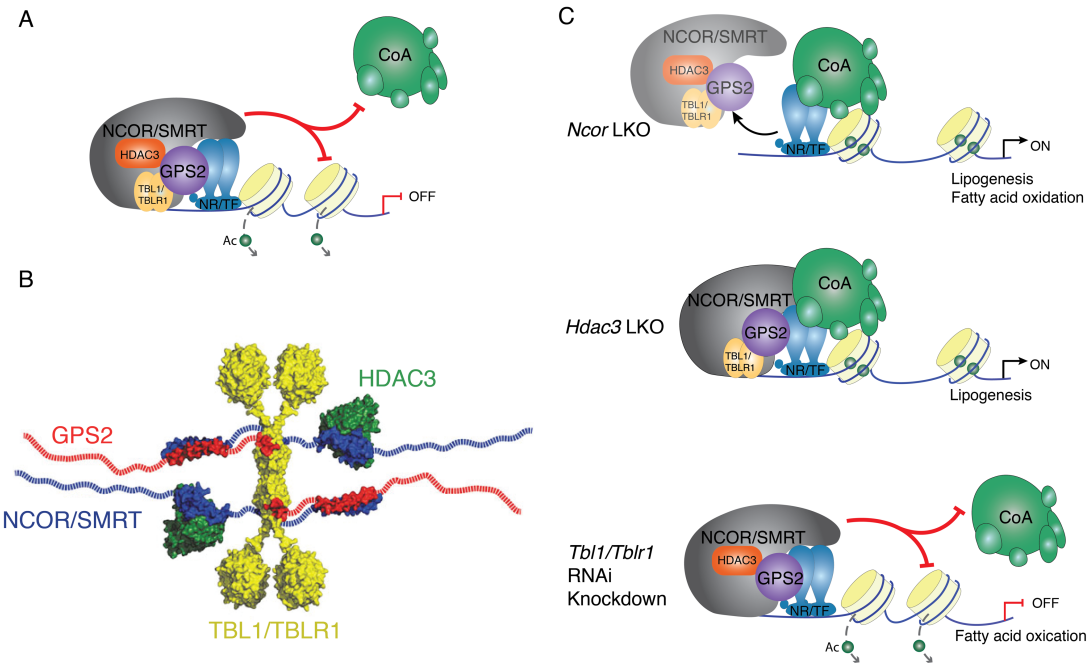

Figure 4 Liver-specific depletion of individual corepressor complex subunits reveals overlapping yet non-redundant roles. In hepatocytes, NCOR, and to a lesser extent SMRT, and GPS2 directly interact with TFs, including metabolite-sensing NRs and inflammatory TFs. HDAC3 requires NCOR interactions for the chromatin recruitment and its repressive function. Highlighted are in (A) the assembly and in (B) the structural information of the core subunits of the corepressor complex in WT hepatocytes. The coiled-coil domain, TBL1/TBLR1 interaction domain and DAD (PDB 1XC5) of NCOR/SMRT are shown in blue. The catalytic domain (PDB 3HGQ) of HDAC3 is shown in green. The N-terminal domain (aa1-90, PDB 1UUJ) and WD40 domain (PDB 2H9M) of TBL1/TBLR1 are shown in yellow. The coiled-coil domain (PDB 2L5G) and TBL1/TBLR1 interaction domain of GPS2 are shown in red. In (C) are the alterations that occur upon depletion of a specific subunit using conditional KO mice or RNAi knockdown in liver. Models are derived from studies that have usually focused on the characterization of one individual subunit, rather than characterizing the entire complex. All highlighted examples are discussed and cited in the text. Ac (acetylation).

Table 1 Summary of loss-of-function mouse models revealing liver-specific corepressor functions and target TFs.

Protein name (gene name)	Mouse model	Key features of the phenotype	Target TF	Ref
GPS2 (*Gps2*)	Global KO	• embryonic lethality around E10		(95)
NCOR (*Ncor1*)	Global KO	• embryonic lethality at E15.5 • impaired erythroid, thymocyte and CNS development		(96)
	LKO (AAV8-TBG-Alb-Cre)	• developed hepatosteatosis due to increased lipogenesis	RevErb LXR	(97)
	LKO (Alb-Cre)	• repressed lipid synthesis in the fasting state • repressed fatty acid oxidation and ketogenesis in the feeding state • improved liver regeneration after partial hepatectomy and blocked diethylnitrosamine (DEN)-induced hepatocarcinogenesis	PPARα LXR ERRα	(98, 99)
	LKO (Alb-Cre)	• developed hepatosteatosis due to increased lipogenesis	TR	(100)
SMRT (*Ncor2*)	Global KO	• embryonic lethality before E16.5 due to lethal heart defect • impaired neural development in forebrain • fail to maintenance of the neural stem cell state	RAR	(101)
	LKO (AAV8-TBG-Alb-Cre)	• no obvious metabolic phenotype	-	(97)
	LKO (Alb-Cre)	• little effect on most of TR targets in either euthyroid or hypothyroid animals • de-repressed RAR targets (*Cyp26a1*)	RAR	(100)
NCOR/ SMRT (*Ncor1/2*)	NCOR/ SMRT LKO (Alb-Cre)	• hepatosteatosis due to activated hepatic lipogenesis and lipid storage • normal glucose sensitivity • increased ChREBP isoforms expression	TR RAR	(100)
	Global NS-DADm transgenic	• upregulated lipid-metabolic genes and mile hepatosteatosis • undetectable HDAC3 enzyme activity, abrogated genome-wide HDAC3 recruitment, as well as increased local histone acetylation level	lipid-sensing NRs	(102)

HDAC3 (*Hdac3*)	Global KO	• embryonic lethality before E9.5		(103)
	LKO (Mx1-Cre plus pIpC injection or Alb-Cre)	• hepatomegaly due to hepatocyte hypertrophy • hepatosteatosis • increased serum TG, total serum cholesterol, and LDL • hypersensitive to insulin	PPARγ2	(104)
	LKO (AAV8-TBG-Alb-Cre)	• alteration in circadian genes • hepatosteatosis due to increased lipogenesis and sequestration • repressed gluconeogenesis • improved insulin sensitivity	RevErb HNF4α HNF6	(97, 102, 105-109)
TBL1 (*Tbl1x*) TBLR1 (*Tbl1xr1*)	Liver RNAi knockdown (adenovirus-delivered shRNA)	• hepatosteatosis • highly elevated VLDL TG • inhibited of PPARα activity under both normal and HFD conditions	PPARα	(110)

1.3.1 Multiple functions of GPS2 in metabolic and inflammatory regulation

GPS2 was initially cloned in 1995 as a human cDNA encoding a potential human suppressor of conserved G-protein pathways in yeast (111). GPS2 was first identified as a NR-associated protein, along with the corepressors receptor-interacting protein 140 (RIP140), NCOR, SMRT, in yeast two-hybrid screenings from liver cDNA libraries using PPARα as bait (112, 113) and later using the orphan receptor small heterodimer partner (SHP) (112). GPS2 was independently biochemically purified as an NCOR/SMRT/HDAC3 corepressor complex subunit and was suggested to be involved in both NR repression and anti-inflammatory crosstalk (88).

GPS2 is a ubiquitously expressed 37 kDa protein, containing 327 amino acid residues (aa). The N-terminal coiled-coil domain (aa 1-90) is sufficient to simultaneously interact with NCOR/SMRT as well as with TBL1/TBLR1, thereby forming a three-way corepressor complex core structure (87). Importantly, GPS2 interacts with several liver NRs (e.g., PPARα, LXRs, SHP) and inflammatory TFs (e.g., c-Jun) by its C-terminal domain (aa 100-327) (82, 112, 114, 115), thus serving as a TF-binding subunit of the corepressor complex, in addition to NCOR and SMRT.

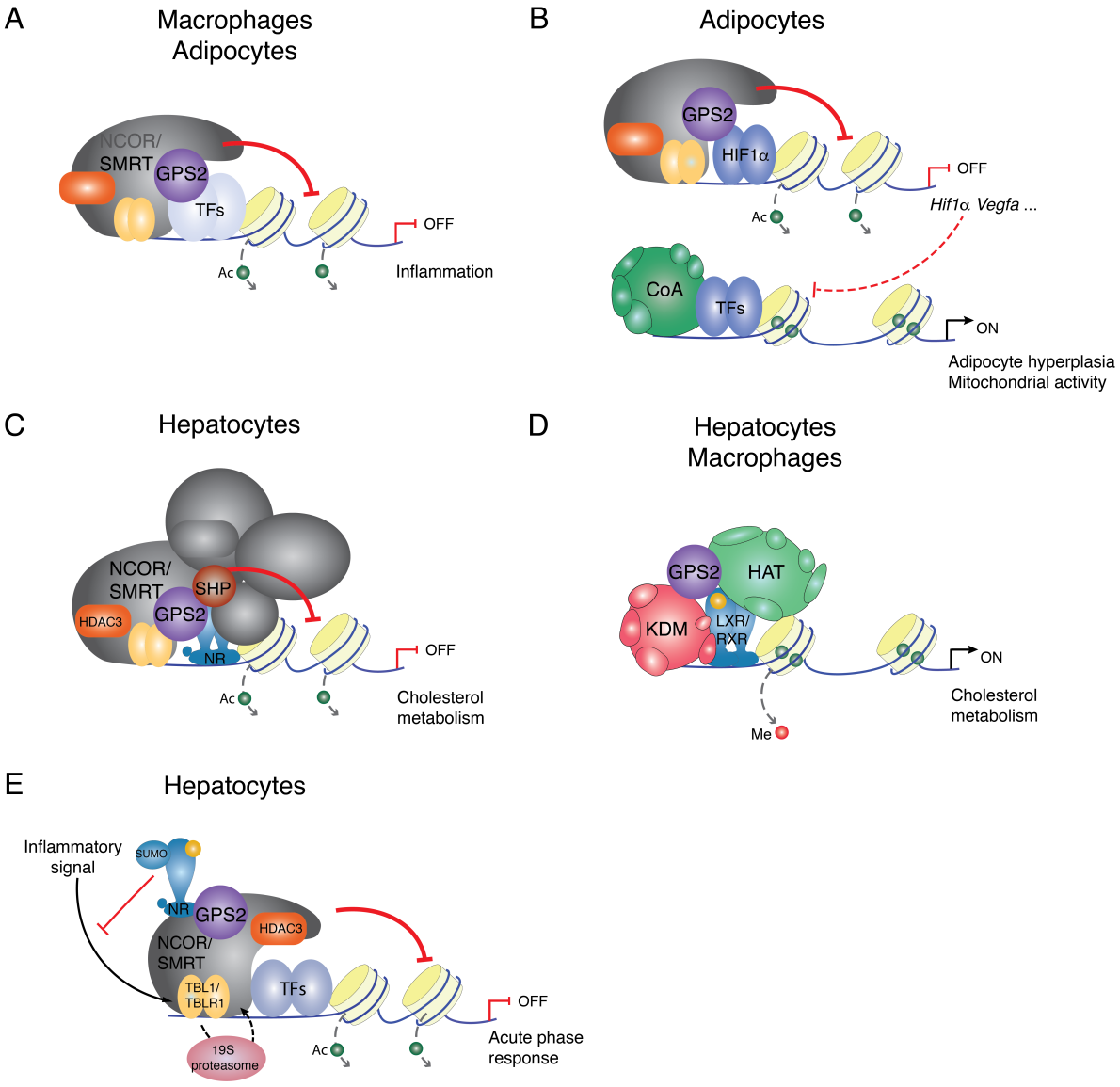

Figure 5 Multiple function of GPS2 in metabolic and inflammatory gene regulation. (A) SMRT/GPS2 corepressor sub-complex cooperates with inflammatory TFs, including c-Jun (in macrophages and adipocytes), NF-κB and C/EBP (in adipocytes), and repress pro-inflammatory gene expression. (B) GPS2 inhibits the activation of HIFα signaling pathway, and maintains the mitochondrial activity and adipocyte hyperplasia. (C) The atypical NR SHP inhibits cholesterol metabolism by acting as a corepressor for multiple NRs via distinct mechanisms, one involving interactions with GPS2. (D) In the presence of activated LXR, GPS2 sequentially recruits KDMs and HATs to prime a suitable chromatin environment for the binding of LXR and coactivators, thus facilitates the LXR-induced ABCG1 expression. (E) GPS2 mediates trans-repression of the hepatic APR by docking to SUMOylated LXR and LRH1. As a consequence, inflammatory signals fail to release the corepressor complex from chromatin and inflammatory gene expression remains repressed. Ac (acetylation).

While metabolic signals that reversibly control GPS2 expression in the liver have not yet been identified, post-translational modifications (PTMs) seem to play critical roles in regulating the protein function of GPS2. Up to now, methylation (R312, R323) (116-118), ubiquitylation (K66) (119) and SUMOylation (K45, K71) (120) of GPS2 have been reported. Further, GPS2 mutations located in the N-terminal coiled-coiled domain were found in the context of human cancers such as medulloblastoma, supporting the role of this domain for appropriate corepressor complex function (121). Whether GPS2 mutations play also a role in human metabolic diseases such as NAFLD is currently not known.

1.3.1.1 NR-independent anti-inflammatory GPS2 actions in macrophages and adipocytes

Both *in vivo* and *in vitro* studies indicate that GPS2 has multiple functions in various aspects of metabolic and inflammatory regulation (86). Many of these functions are consistent with a role of GPS2 as a core subunit of the corepressor complex, while others point to its independent roles in transcriptional activation, gene- and cell-type-selective, and even in non-genomic signaling.

The key anti-inflammatory role of GPS2 as epigenome modifier was identified by macrophage-specific *Gps2* KO (MKO) mice along with genomic investigations in tissue macrophages and in the mouse macrophage RAW cell line (Figure 5A). The study also identifies a GPS2-SMRT sub-complexes as an epigenomic component of metabolic adipose tissue macrophage activation in the context of obesity and T2D (90).

In human adipose tissue, both in adipocytes and infiltrating macrophages, *GPS2* expression was downregulated in obese subjects, and was inversely correlated to the diabetic status and the expression of key inflammatory genes (90, 122). The anti-inflammatory role of GPS2 in adipocytes was further confirmed *in vivo* in aP2-GPS2 transgenic mice (Cardamone et al. 2012). In addition, a cytoplasmic role of GPS2 was suggested to be required for preventing the hyper-stimulation of tumor necrosis factor alpha (TNFα)-induced gene program (*Interleukin 12 beta (Il-12β)* and *C-C motif chemokine ligand 2 (Ccl2*)) (123).

Recent studies using adipocyte-specific *Gps2* KO mice revealed additional pathways to be affected, such as hypoxia-inducible factor 1-alpha (HIF1α) pathways and mitochondrial biogenesis, but the underlying genomic vs. non-genomic mechanisms remain to be clarified (Figure 5B) (124-126). So far it is probably safe to state that both in macrophages and adipocytes GPS2 seems largely, but not exclusively, to cooperate with SMRT to function

within an anti-inflammatory corepressor complex targeting inflammatory and other TFs (Figure 5A), but surprisingly few NRs (e.g., PPARγ-regulated lipolysis in adipocytes).

1.3.1.2 NR-dependent metabolic and anti-inflammatory GPS2 actions in hepatocytes

A few studies have begun to analyze the role of GPS2 in mouse and human hepatocytes. They revealed that GPS2, via interacting with different NRs (such as LXR, FXR, liver receptor homolog-1 (LRH1), HNF4α, and SHP), plays important roles in metabolic and inflammatory regulation of liver pathways, some of which are involved in NAFLD/NASH (82, 112, 114).

The two initial studies revealed that GPS2 serves as a physiological coregulator of cholesterol homeostasis by affecting cholesterol to BA biosynthesis in the liver (112) and by participating in cholesterol transport and efflux in hepatocytes and macrophages via ABCG1 (114). Activation of FXR initiates a feedback regulatory loop via induction of the orphan receptor and corepressor SHP, which suppresses LRH1- and HNF4α-dependent expression of *CYP7A1* and *CYP8B1*, the two major enzymes for BA synthesis. The first study suggested a model in which GPS2 regulates these genes by two separate mechanisms in opposite ways. At the *CYP7A1* promoter, GPS2 may serve as a bridging protein to connect the the corepressor complex with SHP, thereby triggered repression (Figure 5C). However, at the *CYP8B1* enhancer and promoter, GPS2 seems required for the recruitment of coactivators, thereby triggering activation (112). The second study found that GPS2 is selectively required to facilitate LXR-induced *ABCG1* expression in human hepatocytes (Figure 5D), while having no effect on LXR-induced *ABCA1* expression (114). This highlights the fundamental molecular differences between transcriptional regulatory elements of two related key LXR target genes encoding cholesterol transporters. By dismissing G9a and recruiting histone lysine demethylases (KDMs) and histone acetyltransferases (HATs) to trigger H3K9 demethylation and subsequent H3 acetylation, GPS2 may prime an appropriate local chromatin environment to facilitate ligand-induced LXR recruitment and promoter-enhancer communication (114). Since GPS2 in that mechanism promotes the chromatin access of a target TF, it may exert an unusual ‘pioneer-type’ function, distinct from classic coactivators. Indeed, a highly related GPS2 mechanism was identified subsequently also for PPARγ in adipocytes (Cardamone et al., 2014).

The anti-inflammatory role of GPS2 can be exemplified by GPS2-dependent actions of LRH1 and LXRβ in trans-repression of the hepatic APR (82). Importantly, GPS2 was identified as a sensor of SUMOylated LRH1 and LXRβ in hepatocytes. This provided the missing link in

the trans-repression model, explaining how the NCOR-containing corepressor complex can be recruited to NRs in the active/liganded conformation. Specifically, the study demonstrated that the GPS2-SUMO-NR complex maintains repression even upon IL-1β and IL-6 stimulation, thereby inhibiting APR gene expression during inflammation and infection (Figure 5E). This study also revealed that in *Sumo-1* KO mice the APR was increased, which may be caused at least partially by diminished LRH1 SUMOylation (82). In sum, these GPS2-focussed studies have conceptually advanced our understanding of multiple functions of GPS2 as well as the individual roles of each subunit of the corepressor complex (86).

1.3.2 NCOR but not SMRT is critical for repression of metabolic pathways in hepatocytes

NCOR (127, 128) and SMRT (129-131) were first identified based on their ability to interact with and repress the activity of unliganded NRs. Both NCOR and SMRT are extremely large proteins (molecular weight around 270 kDa), which is suitable for forming a scaffold-binding surface for simultaneous interactions with target TFs, other coregulators and histone modifiers to form a corepressor complex.

GPS2 and TBL1/TBLR1 bind with each other directly, and simultaneously they both interact with distinct conserved regions of the NCOR/SMRT N-terminal repression domains (RD), forming a three-component complex (87, 88, 94). HDAC3 directly binds to the deacetylase-activation domain (DAD) of NCOR/SMRT (88, 132). The DAD binding is critical for recruitment and activation of HDAC3 (102, 132, 133). Receptor-interaction domains (RIDs), at the C-terminal regions of NCOR and SMRT, have been identified to interact with the ligand binding domains of unliganded NRs (128, 134).

Global genetic deletion of *Ncor* (96) or *Smrt* (101) both causes embryonic lethality. Therefore, to characterize the *in vivo* functions, global or liver-specific transgenic mouse models carrying different mutations or deletions of NCOR and SMRT have been developed (102, 135-145).

Liver depletion of NCOR, not SMRT, causes hepatosteatosis

The *Ncor* LKO liver phenocopied *Hdac3* LKO metabolic changes, displayed increased hepatic lipid accumulation, and the reciprocal reduction of hepatic glycogen content (Figure 4C). Consistent with the lipid metabolic phenotypes, the transcriptome profiling of *Ncor* LKO and *Hdac3* LKO livers also showed high similarity, as the upregulated genes were both greatly enriched in lipid and fatty acid metabolism (104, 107). In contrast, unlike the

hepatosteatosis phenotype caused by the depletion of liver NCOR or HDAC3, liver SMRT depletion showed no obvious changes in the context of lipid metabolism (97). Genome-wide chromatin occupancy of NCOR, but not SMRT, revealed a robust circadian rhythm in phase with HDAC3, suggesting that NCOR plays a more important role than SMRT in the genomic recruitment of HDAC3 in liver (97, 105).

NCOR represses PPARα-induced fatty acid oxidation and ketogenesis

In addition, S6 kinase 2 (S6K2), a downstream effector of mammalian target of rapamycin (mTORC1) signaling pathway, interacts with NCOR and promotes its nuclear localization, which then represses PPARα activity in hepatocytes (146, 147). In line with this, S6K2 has markedly elevated activity in *ob*/*ob* mice, a genetic mouse model of obesity, and NCOR is predominantly localized in hepatocyte nuclei with S6K2. Thus, this reveals a mechanism for how energy availability may direct influence the nuclear localization of a key corepressor, thereby PPARα repression and hepatic ketogenesis (146).

Signal-regulated phosphorylation of NCOR

NCOR repressed lipogenic genes expression as enhanced hepatic lipogenesis and lipid storage were observed in *Ncor* LKO mice (97, 135, 137, 144). In addition, NCOR also repressed PPARα-induced hepatic FAO and ketogenesis (146, 147). These apparently paradoxical observations indicate that NCOR may select its TF targets in a context-dependent manner according to the cellular energy status to orchestrate liver energy metabolism. Indeed, the insulin-Akt signaling pathway differentially modulates NCOR activity by inducing phosphorylation at serine 1460 during the feeding-fasting transition. Phosphorylated NCOR selectively prefers to interact with PPARα and estrogen-related receptor alpha (ERRα) over LXRα, and this leads to a de-repression of LXRα target genes to increase lipogenesis whilst inhibits PPARα and ERRα target genes to attenuate oxidative metabolism in the liver. Therefore, *Ncor* LKO concurrently induces both lipogenesis and oxidative metabolism due to a global de-repression of LXRα, PPARα, and ERRα activity (Figure 6).

More generalized, PTMs such as phosphorylation could provide an important mechanism by which corepressors can switch targets and selectively modulate liver metabolism (98). Indeed, multiple phosphorylation sites, some of which are regulated by the insulin-signaling, have been identified in NCOR and other corepressor complex subunits in the context of mouse liver steatosis and insulin signaling (148).

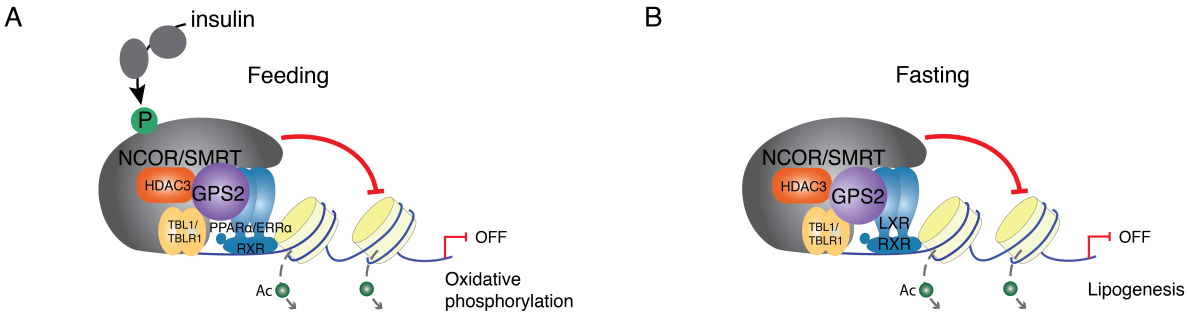

Figure 6 Phosphorylation of the NCOR by the insulin-Akt signaling pathway switches its corepressor targets in the liver. NCOR selects its targets depending on the cellular energy status. Insulin induces phosphorylation of NCOR serine 1460, which selectively favors NCOR interaction with PPARα and ERRα over LXRα. As a result, PPARα/ERRα-dependent oxidative metabolism is attenuated due to repression during feeding (A), while LXRα-dependent lipogenesis is repressed during fasting (B). Ac (acetylation).

1.3.3 HDAC3 controls circadian rhythm and physiology

All the mammalian HDAC superfamily members have a highly conserved deacetylase domain. Only HDAC3 is exclusively found in the endogenous NCOR/SMRT corepressor complex, suggesting that HDAC3 is the enzyme responsible for histone deacetylation at regulatory promoters and enhancers that are controlled by this particular complex. Its deacetylase activity requires interactions with NCOR/SMRT (91). Thus, HDAC3 could be particularly important for connecting the transcriptional and epigenetic functions of the complex in the liver.

Hdac3 LKO mice revealed the pivotal role of HDAC3 in the regulation of the circadian rhythm as well as of hepatic lipid, cholesterol and carbohydrate metabolism (104, 105, 107). HDAC3 depletion caused severe liver steatosis, with dramatically elevated hepatic and serum TG and cholesterol levels, due to increased DNL and cholesterol synthesis but decreased FAO (104, 105) (Figure 4C). However, because the intermediary metabolites were rerouted from hepatic gluconeogenesis to DNL and the subsequent sequestration, the *Hdac3* LKO mice concurrently showed improved glucose tolerance and insulin sensitivity (107).

Two core components of the circadian clock, the NRs RevErbα and RevErbβ, were found to fully account for the circadian rhythmicity of NCOR-HDAC3 occupancy (106, 108) (Figure 7A and B). Consistently, both the RevErbα- and the NCOR-depleted mice exhibit liver steatosis, identical to the *Hdac3* LKO phenotype (97, 105). Notably, the *Hdac3* LKO mice had much more severe liver steatosis than those lacking the two RevErbs (106), suggesting

that the NCOR-HDAC3 sub-complex may interact with additional TFs to control the expression of genes involved in lipid homeostasis independently of RevErb.

A recent liver HDAC3 interactome analysis identified the previously known NR corepressor and homeodomain TF prospero homeobox protein 1 (PROX1) as an HDAC3-associated factor (109). HDAC3 and PROX1 co-occupied extensively at regulatory regions of metabolic genes, and liver depletion of PROX1 increased hepatic TG, mimicking *Hdac3* LKO phenotype. The HDAC3-PROX1 module was recruited to the genome by HNF4α, rather than RevErb, because their chromatin recruitment was remarkably reduced upon depletion of HNF4α in hepatocytes and REV-DR2 motif was only present in HDAC3-specific peaks (109) (Figure 7C).

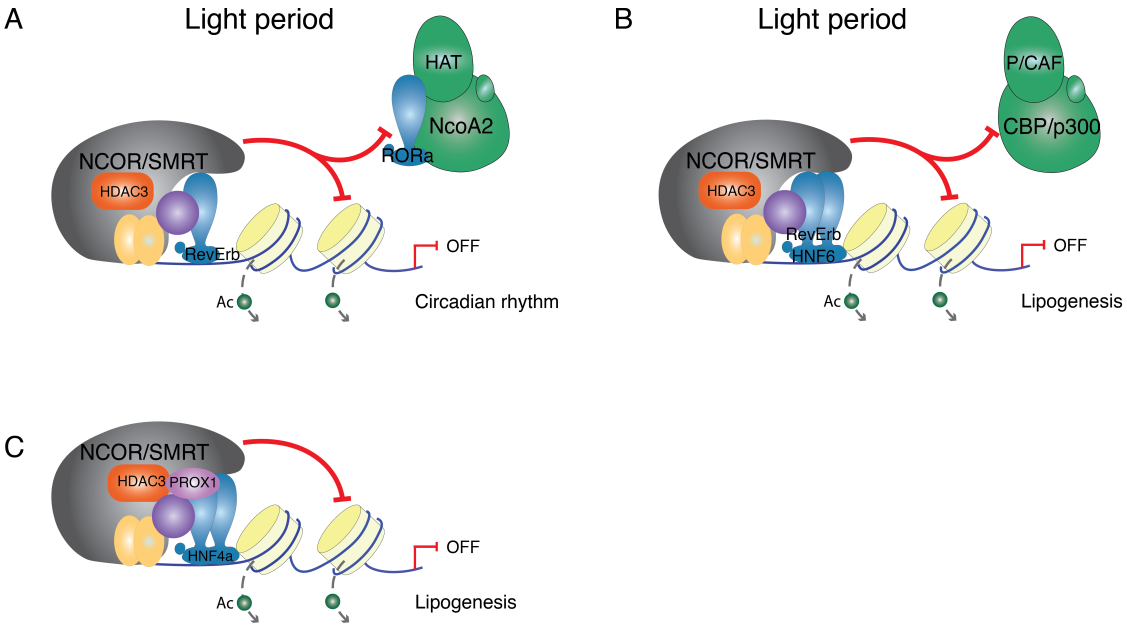

Figure 7 HDAC3 controls both circadian rhythm and physiology. (A) RevErbα recruits the NCOR-HDAC3 complex to its canonical RevDR2 DNA-binding motifs, competing with RORα and the associated coactivator complex, to repress circadian clock genes during the light period. (B) Simultaneously, HNF6 recruits RevErbα and the associated corepressor complex to mediate circadian repression of lipogenic gene expression during the light period. (C) Immuno-precipitation-coupled mass spectrometry from mouse liver extracts identified interactions of the HDAC3 complex with the homeodomain corepressor PROX1, which mediates the recruitment of HDAC3/NCOR to HNF4α-bound enhancers to repress lipogenesis. Ac (acetylation).

Another study revealed an intriguing role of HDAC3 in the age-dependent epigenome reprograming linked to metabolic dysfunction (149). In the livers of older mice (21 month), loss of HDAC3 chromatin occupancy caused increased histone acetylation and triggered the gain of forkhead box protein A2 (FOXA2) at the regulatory elements, which further

cooperated with PPARα to upregulate genes involved in lipid synthesis and storage. This reciprocal binding of FOXA2 and HDAC3 contributes to the de-repression of PPAR and LXR and thereby triggers aging-related liver steatosis (149).

Although deacetylase enzyme activity of HDAC3 was barely detectable in (NCOR/SMRT) DADm mice, the DADm mice failed to phenocopy the dramatic effects of *Hdac3* LKO on hepatic lipid metabolism (102, 105). This indicates that the HDAC3 enzymatic activity is only part of the mechanisms for the key repressive functions of the corepressor complex (97). In addition, several deacetylase-dead HDAC3 mutants (Y298A, H134A/H135A, and K25A) could largely reverse the liver steatosis in *Hdac3* LKO mice (97, 102). Only the HEBI mutant of HDAC3, whose interaction with NCOR/SMRT was completely abolished, failed to rescue the *Hdac3* LKO phenotype. Therefore, the repressive function of HDAC3 in the liver, albeit independent of deacetylase activity, totally depends on the interaction with NCOR, while SMRT seems dispensable. Thus, HDAC3 functions not independently but only as a subunit of the NCOR corepressor complex (97).

1.3.4 TBL1 and TBLR1 regulate fatty acid oxidation in liver

Structure data suggest that TBL1 and its homologue TBLR1 directly interact with the NCOR/SMRT RID domain and GPS2 via distinct N-terminal regions (87, 94).

Upon ligand treatment, unlike the dismissal of NCOR-HDAC3, TBL1/TBLR1 could still be recruited together with the coactivators. Mechanistically, TBL1/TBLR1 serve as NR corepressor-coactivator-exchange factors (N-CoEx), which in the presence of ligands recruit the ubiquitin conjugating/19S proteasome complex for the ubiquitination and dismissal of the corepressor complex, and facilitate the coactivator recruitment (150, 151).

To identify the physiological role of TBL1 and TBLR1 in liver, TBL1/TBLR1-depleted mouse models were established. Depletion of hepatic TBL1 or TBLR1 resulted in significantly increased hepatic TG, serum VLDL TG levels and decreased serum ketone bodies levels. Simultaneous depletion of both TBL1 and TBLR1 triggered a much more severe phenotype than individual depletion, and these effects were gone in *Pparα* KO mice. Consequently, all the results demonstrated that TBL1 and TBLR1 synergistically prevent liver steatosis and hypertriglyceridemia by regulating FAO genes in a PPARα-dependent manner (110) (Figure 4C). In line with the N-CoEx function, TBL1/TBLR1 depletion triggered the release of known PPARα coactivators and promoted the recruitment of NCOR and HDAC3 to the FAO promoters (110).

Surprisingly, the TBL1/TBLR1-depleted mice display mildly improved systemic glucose tolerance and insulin sensitivity even with severe liver steatosis, similar to the *Hdac3* LKO phenotype. This suggests that TBL1/TBLR1 may regulate liver lipogenesis as part of the corepressor complex (107, 110).

The coactivator function was independently demonstrated in a previous study which demonstrated the requirement of the corepressor core subunits for LXR activation in human hepatocytes and macrophages. It was found that depletion of TBLR1 but not of TBL1 reduced ligand-dependent LXR activation of key target genes (114). Interestingly, TBLR1 seems to cooperate with GPS2 in this LXR pathway, although at mechanistically distinct steps. Therefore, the liver studies confirm that TBL1 and TBLR1, unlike the other core subunits of the corepressor complex, function as coactivators for multiple LXR and PPARα target genes.

2 AIMS OF THIS THESIS

The objective of this thesis is to understand the roles and relationship of individual TFs/NRs and GPS2, along with the corepressor complex, in the development of obesity-related metabolic disorders, including NAFLD and cardiovascular diseases. Within three major aims the presented studies should advance our knowledge of the regulatory networks and mechanisms underlying the development of metabolic disorders:

AIM 1. To investigate the functions of GPS2 in hepatocytes on lipid metabolism and on NAFLD/NASH progression through *Gps2* LKO mice and through the analysis of gene expression datasets in humans.

AIM 2. To characterize the involvement of GPS2 in LPS-regulated cholesterol efflux through *Gps2* MKO mice and through *in vitro* studies using RAW cells.

AIM 3. To explore the role of LXRα phosphorylation in hepatic lipid metabolism during NAFLD progression through LXRα-S196A knockin mice.

3 METHODOLOGICAL CONSIDERATIONS

Detailed materials and methods are described in each paper included in this thesis, and this chapter aims to highlight specific aspects about some of the methods used.

3.1 Patients

The patient clinical information and human liver biopsy transcriptome data are from a previously published Belgium cohort (26) collected from overweight individuals visiting the Obesity Clinic at the Antwerp University Hospital. The patient information and exclusion criteria were all described previously (26). Briefly, the cohort used in this study is composed of 104 NASH patients with paired liver biopsies of 35 samples after dietary intervention and 39 samples after GABY (combined as weight loss group). The fibrosis stage was determined by pathological analysis of the liver biopsies. BMI (loss), HbA1c, HDL-c and LDL-c were determined as described previously (26), and the study was approved by the Ethical Committee of the Antwerp University Hospital (file 6/25/125).

3.2 Mouse models

***Gps2*$^{flox/flox}$ mice** were generated at Ozgene Pty, Ltd. (Bentley DC, Australia) using a targeting construct, which contained loxP sites flanking exons 2 and 5, followed by a FRT site and a neomycin cassette inserted between exons 5 and 6 (90). The targeting vector was electroporated into C57BL/6 Bruce4 embryonic stem (ES) cells. The correctly recombined ES colony was then injected into C57BL/6 blastocysts. Male chimeras were mated with female C57BL/6 mice to get mice with a targeted Gps2 allele. The mice were crossbred with C57BL/6 flp-recombinase mice to remove the neomycin cassette to create heterozygous *Gps2*$^{flox/+}$ mice. The mice were then crossbred with C57BL/6 mice for nine generations before being bred with heterozygous *Gps2*$^{flox/+}$ mice to get the *Gps2*$^{flox/flox}$ mice. To generate ***Gps2* LKO mice**, *Gps2*$^{flox/flox}$ mice were crossed with Alb-*Cr*e mice (B6.Cg-*Speer6-ps1*$^{Tg(Alb-cre)21Mgn}$/J; Jackson Laboratory stock no. 003574). *Gps2*$^{flox/flox}$ mice from the same breedings were used as control (labeled as WT).

***Pparα* KO mice** (152) (B6. 129S4-Ppar*α*^{tm1Gonz}/J; Stock no. 008154) were obtained from Jackson Laboratory.

Liver-specific *Gps2* and *Pparα* double KO (DKO) mice (*Gps2*$^{flox/flox}$ Alb-*Cre*$^{+/-}$*Pparα*$^{-/-}$) were generated by breeding the liver *Gps2* KO mice with the *Pparα* KO mice. *Gps2*$^{flox/flox}$Alb-*Cre*$^{-/-}$*Pparα*$^{-/-}$ mice were used as control.

Liver-specific *Ncor* and *Smrt* KO mice were generated and maintained in Anthony Hollenberg's lab as previously described (100). Generally, *Ncor*$^{flox/flox}$ and *Smrt*$^{flox/flox}$ were crossed with Alb-*Cre* mice (B6.Cg-*Speer6-ps1*$^{Tg(Alb-cre)21Mgn}$/J; Jackson Laboratory stock no. 003574). *Ncor*$^{flox/flox}$ and *Smrt*$^{flox/flox}$ mice from the same breedings were used as respective control.

LXRα-S196A mice were generated and maintained in Inés Pineda-Torra's lab. The S196A$^{flox/flox}$ mouse line was generated by Ozgene Pty Ltd (Bentley WA, Australia). The mutant fragment, located on Exon 5, contains a serine-to-alanine mutation at Ser196 introduced by site-directed mutagenesis. The point mutant exon was delivered into an intronic site inside the targeting vector, placed in opposite orientation and thus without coding capacity. The targeting construct was electroporated into the Bruce4 C57BL/6 ES cell line. Homologous recombinant ES cell clones were identified by Southern hybridization and injected into BALB/cJ blastocysts. Male chimeric mice were obtained and crossed to C57BL/6J females to establish heterozygous germline offsprings on a pure C57BL/6 background. The germline mice were crossed to a FLP Recombinase mouse line (153) to remove the FRT flanked selectable marker cassette (Flp'd mice). Flp'd mice were then crossed with a transgenic C57BL/6 mouse strain carrying a Cre recombinase under the PGK-1 promoter (154), resulting in the inversion and insertion of the lox-flanked mutated (loxP) vector exon 5 region in the sense orientation, and deletion of the WT sequence in most adult cell lineages (S196A mice) while WT matching controls carry the WT sequence in the sense orientation.

All animals were randomly assigned to each experimental group. All animal experiments were approved by the respective national ethical boards and conducted in accordance with the guidelines stated in the International Guiding Principles for Biomedical Research Involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS). *Gps2* LKO, *Pparα* KO and *Gps2-Pparα* DKO as well as their respective control mice strains were bred and maintained at the Center for Comparative Medicine at Karolinska Institutet and University Hospital (PKL, Huddinge, Sweden).

3.3 *Gps2* KO RAW cell line

The RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system, and have been developed as efficient genome editing tool to generate modified cell lines for downstream functional studies (155). We applied this technology to delete coding DNA sequences of Gps2 in RAW 264.7 cells. To minimize the off-target effects, we chose double-nicking strategy using the nickase mutated Cas9 with paired single guide RNAs (sgRNAs). The paired sg RNAs were designed on CRISPR gRNA design tool Design 2.0. The following sequences of sgRNAs (sgRNA1: CACCGGCAAACGGCAGGGTGAGCCT and sgRNA2: CACCGGATGTGCCGGTGCAGAGCCC) were inserted into PX461 vector65 (Addgene plasmid 48140) and sorted by FACS. The single-cell colonies were validated via sequencing for at least five separate T clones, and were further validated by western blot. PX461 empty-vector transfected and sorted single-cell clones were used as negative controls.

3.4 Microarray analysis

Raw-intensity expression files (.CEL files) were imported to R and Bioconductor using the Oligo package (156). The same package was used for quantile normalization, background correction, and summarization by robust multichip average preprocessing (RMA). The normalized log2-transformed expression values were then imported to the Limma (157) package for differential-expression analysis by linear modeling. A paired design was used to remove the batch effect between the biological replicates. Furthermore, genes with low expression (less than the 95th quantile of the negative-control probes) were removed. Genes with a P value of <0.05, after adjusting for multiple hypothesis testing using the FDR method, were defined as being differentially expressed.

Correlation matrix was calculated based on expression values in log2 scale of 251 candidate NASH genes (26) and GPS2-NCOR-HDAC3 complex components from 104 NASH livers at baseline. Genes whose expression significantly correlated with *GPS2* expression were selected and their correlation with GPS2-NCOR-HDAC3 complex components was plotted as heatmap.

3.5 RNA-seq and data analysis

RNA was extracted from mice liver biopsies as described above. RNA quality was assessed by 2200 TapeStation Instrument (Agilent). PolyA RNA selection was performed using the Illumina TruSeq RNA Sample Preparation Kit according to the manufacturer's protocol.

RNA-seq libraries were prepared and sequenced on the Illumina HiSeq 2000 platform at Bioinformatics and Expression Analysis core facility (BEA, Karolinska Institutet, Sweden). Preprocessed reads were aligned to the mm9 transcriptome using the HISAT2 program (158), and HOMER (Hypergeometric Optimization of Motif EnRichment, http://homer.salk.edu/homer) (159)was used to create the tag directory and count tags in all exons. Raw tag counts were imported in to R and Bioconductor and edgeR package was used to determine differential gene expression (160). Ggplot2 package was used for the dot plot (MA plot and volcano plot) and box plot (161).

3.6 ChIP-seq sample preparation

ChIP-seq was performed using both fresh and frozen livers, with protocols modified in each condition (90, 162). Briefly, fresh livers were chopped into small pieces and frozen livers were pulverized into powder before subjected to crosslinking. Then the liver pieces were crosslinked with 1% formaldehyde (ThermoFisher, 28906) in PBS for 10 min for histone modifications, or double crosslinked with 2 mM disuccinimidyl glutarate (DSG) for 30 min, followed by 1% formaldehyde for 10 min, for TFs and GPS2. The reaction was stopped with glycine at a final concentration of 0.125 M for 5 min. Crosslinked liver pieces were disaggregated in ice-cold PBS with protease inhibitor using Dounce Homogenizer first with loose and later with tight pestle (Fisher Science, FB56691). Nuclei were isolated using lysis buffer 1 (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% IGEPAL CA-630 and 0.25% Triton X-100), lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA), and lysis buffer 3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% N-Lauroylsarcosine), and subsequently sonicated for 30 min (30 s ON/ 30s OFF) in the Bioruptor Pico (Diagenode). Protein A Dynabeads (Invitrogen) were incubated O/N with the antibodies. Each lysate was immunoprecipitated with the following antibodies: anti-H3K4me3 (Abcam, ab8580, 1 μg), anti-H3K27ac (Abcam, ab4729, 1 μg), anti-PPARα (Millipore MAB3890, 5 μg), anti-Polymerase II (Biolegend, 664906, 5 μg), anti-NCOR (Bethyl laboratories, A301-145A, 4 μg), anti-SMRT (Bethyl laboratories, A301-147A, 4 μg), anti-HDAC3 (Santa Cruz, sc-11417, 5 μg) and anti-GPS2 (4 μg). Formaldehyde crosslinking was reversed overnight at 65 °C, and the immunoprecipitated DNA was purified using the ChIP DNA Clean & Concentrator Capped Zymo-Spin I (Zymo Research) purification kit.

For library preparation and sequencing, 2–10 ng of ChIPed DNA was processed using Rubicon ThruPLEX DNA-seq kit (TAKARA) or processed at the EMBL Genomics Core

Facility (Heidelberg, Germany) using standard protocols, and sequenced in the Illumina HiSeq 2000 (50SE reads, EMBL) or NextSeq 550 (75SE reads, Bioinformatics and Expression Analysis (BEA, Karolinska Institutet, Sweden) Core Facility).

3.7 ChIP-seq data analysis

Raw sequencing files (fastq files), our own sequenced or the published ChIP-seq data from European Nucleotide Archive (ENA), were aligned to the NCBI37/mm9 version of the mouse reference genome, using Bowtie2 with default settings (163). Duplicated reads were removed when using HOMER (159) makeTagDirectory program to generate tag directories. Peaks were determined (more than 4-fold enrichment over input and local tag counts and FDR less than 0.001) by the HOMER findPeaks program against the input samples. Bedgraph files were generated by HOMER makeUCSCfile program. Peak heights were normalized to the total number of uniquely mapped reads and displayed in IGV (Integrative Genomics Viewer) (164) as the number of tags per 10 million tags. The sequences found in GPS2 peaks were subjected to motif analysis to identify potentially over-represented TF-binding sites using Homer findMotifsGenome program. To generate ChIP-seq heatmap, Homer annotatePeaks program was used first to generate the data matrix of read density of each ChIP-seq sample in the ±3kb (from the center of the peak) window with 25 bp bin size. The clustering was performed in Cluster 3.0 (165) using self-organizing maps, and then visualized in TreeView (166). For statistical analysis of the peaks, raw tag counts were imported in to R and Bioconductor. Normalization was performed first based on total sequencing tag counts, rather than total tag counts in peaks. The edgeR package was then used to calculate fold change as well as p. value based on the normalized tag counts (160, 167). Ggplot2 package was used for the dot plot (MA plot and volcano plot) and box plot (161).

4 RESULTS

4.1 PAPER I: Hepatocyte-specific loss of GPS2 in mice reduces NASH via activation of PPARα

Alterations of regulatory transcription networks and epigenomes in hepatocytes are involved in obesity-triggered NAFLD development. The phenotypes of LKO mice depleting individual corepressor complex subunits have been linked to multiple target TFs, resulting in the modulation of partially opposing liver metabolic pathways. In this study, we combined *Gps2* LKO mice with human transcriptome datasets analysis and uncovered a hitherto unknown role of GPS2 as an epigenetic modulator in hepatocytes that represses PPARα-dependent lipid catabolism and thereby promotes the progression of NAFLD/NASH in both mice and humans.

4.1.1 *Gps2* LKO improves MCD-induced fibrosis in mice

To explore the function of GPS2 *in vivo*, we generated *Gps2* LKO mice. Intriguingly, *Gps2* LKO presented a dramatical reduction in serum VLDL and total TG level, due to the increased lipid oxidation as detected by the increased ketone body production.

Then we challenged the mice with MCD to induce liver fibrosis. The LKO mice exhibited improved liver steatosis and fibrosis, as evidenced by the reduced serum ALT and AST, hepatic TG and collagen fiber content, and macrophage infiltration. Consistently, expression of fibrotic and inflammatory markers decreased in LKO livers upon MCD, suggesting that *Gps2* depletion in hepatocytes improves MCD-induced inflammation and fibrosis indirectly via enhancing fatty acid metabolism.

4.1.2 *Gps2* LKO improves liver steatosis and insulin resistance

We further challenged the mice with HFD to explore the role of hepatocyte GPS2 in obesity-induced liver steatosis and insulin resistance. HFD fed LKO mice gained less body weight, showed improved liver steatosis and better glucose control. In addition, serum VLDL and total TG levels were reduced in HFD fed LKO mice, due to the reduced lipogenesis gene and increased FAO gene expression. Thus, our results suggest that hepatocyte GPS2 plays a key role in modulating both lipid metabolism and glucose homeostasis in obese mice.

4.1.3 PPARα is a direct target of GPS2 in hepatocytes

To further investigate the underlying mechanisms involved in the regulatory role of GPS2 in the liver, we performed RNA-seq to identify the transcriptome changes and in parallel GPS2 ChIP-seq for the genome-wide GPS2 occupation, using WT and LKO livers from CD fed mice. KEGG pathway analysis revealed the enrichment of PPAR signaling pathways in GPS2 repressed genes. The up-regulation of PPARα genes (e.g., *Pyruvate dehydrogenase lipoamide kinase isozyme 4* (*Pdk4)*, *Cyp4a14* and *Fgf21*) in LKO livers were further upregulated in fasted and GW7647-treated (a PPARα agonist) mice. To demonstrate the requirement of PPARα in the GPS2 mediated repression of lipid metabolic genes, *Gps2* and *Pparα* double KO (PGKO) mice were generated. Not surprisingly, the fatty acid oxidative effects of GPS2 depletion in the liver disappeared in the absence of PPARα. Collectively, these data identify PPARα as a major target for GPS2 in the liver.

The integrated analysis revealed that the GPS2-dependent transcriptome (RNA-seq) and epigenome (H3K4me3 and H3K27ac ChIP-seq) changes were highly correlated genome-widely as well as at representative gene loci (e.g., *Pdk4* and *Cyp4a14*). Moreover, the increased H3K27ac at *Pdk4* and *Cyp4a14* promoter/enhancer loci upon GPS2 depletion was not observed in PGKO versus PKO mice, suggesting that PPARα is required for GPS2-mediated epigenomic repression at those gene loci.

4.1.4 GPS2 cooperates with NCOR in hepatocytes

The apparent PPARα-selectivity of GPS2 repression was surprising as it was unique in comparison with LKO models for other corepressor complex subunits. To investigate whether GPS2 functions within the corepressor complex or not, we first compared the chromatin occupation of NCOR and SMRT with GPS2 in mouse livers, and the transcriptome signatures from respective KO livers. Cistrome analysis showed that all three subunits shared more than one half of all peaks in the liver genome. However, PPAR signaling pathways were only enriched in GPS2 and NCOR, but not GPS2 and SMRT, corepressed genes. In addition, GPS2 recruitment was only abolished in observed in *Ncor*, but not *Smrt*, LKO livers. Based on these results, we concluded that GPS2 cooperates with NCOR in mouse hepatocyte.

Next, we determined genome-wide chromatin occupancy for GPS2, NCOR, and PPARα, along with H3K27ac enhancer mark, in the respective KO livers comparing with WT livers. Intriguingly, we found that (i) loss of PPARα caused the release of GPS2 and Ncor from chromatin, (ii) loss of NCOR caused the release of GPS2 and the recruitment of PPARα, (iii)

loss of GPS2 led to an increased PPARα recruitment, despite not being sufficient for causing NCOR release from chromatin. Moreover, the *in vitro* co-immunoprecipitation results showed that full-length GPS2, but not truncated GPS2 variants without the NCOR-interaction domain or the PPARα-interaction domain, could enhance the NCOR-PPARα interaction. These data supported the hypothesis that the presence of GPS2 in hepatocytes stabilizes interactions of PPARα with the NCOR complex to repress FAO gene expression.

4.1.5 Liver *GPS2* expression correlates with NASH fibrosis in humans

We also confirmed clinical relevance of GPS2 in the progression of NAFLD in human patients, because we found that *GPS2* expression positively correlated with fibrogenic genes, including *TGFB* and *TIMP1*, in both NAFLD and NASH human subjects. Although there was no difference between different NASH stages, *GPS2* expression was higher in fibrosis NASH than non-fibrosis NASH liver biopsies and was restored after weight loss therapy in paired obese human subjects. Therefore, liver GPS2 expression and function might be causatively correlated with the progression of NAFLD towards NASH fibrosis via regulating PPARα-coupled liver lipid metabolism (Figure 8).

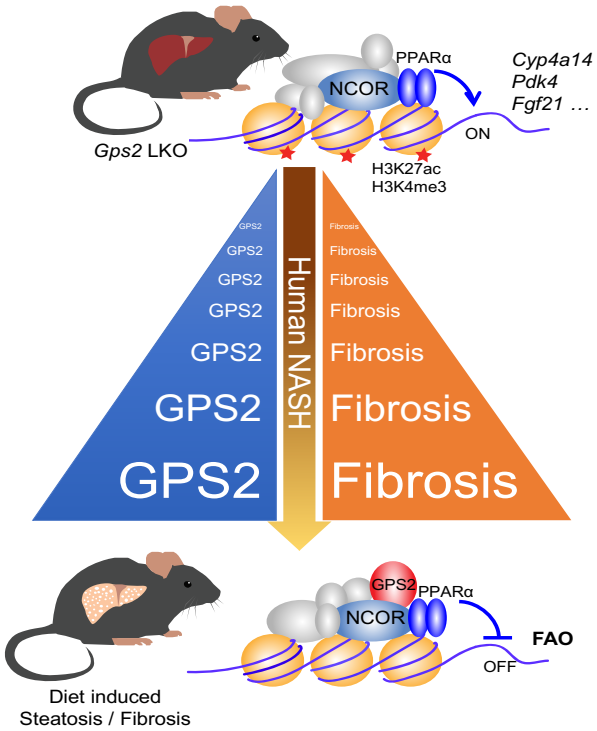

Figure 8 Model illustrating the causal relationship between GPS2 expression and PPARα function in hepatocytes in the context of fatty liver disease.

Reprinted from "Liang, N., et al. (2019). "Hepatocyte-specific loss of GPS2 in mice reduces non-alcoholic steatohepatitis via activation of PPARalpha." Nature Communications 10(1): 1684. doi: 10.1038/s41467-019-09524-z" under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)

4.2 PAPER II: GPS2 links inflammation and cholesterol efflux by controlling LPS-induced ABCA1 expression in macrophages

Macrophages play substantial roles in linking alterations of cholesterol metabolism and inflammation to the development of atherosclerosis. Previous studies have identified several crosstalk mechanisms that connect cholesterol efflux and inflammation. It has also been demonstrated that ABCA1, a main regulator of cholesterol efflux, is specifically necessary for the anti-inflammatory effects of LXR ligands. However, the extent to which these pathways influence each other has only partly understood.

To specifically investigate the possible roles of GPS2 in ABCA1 regulation, CRISPR/Cas9-mediated KO and lentivirus-mediated RNAi knockdown methods were applied to both mouse and human macrophage cell lines. Consistent with previous findings (90), GPS2 deletion significantly up-regulated pro-inflammatory gene expression (e.g., *Ccl2* and *Ccl7*) upon LPS treatment. More importantly, we found that GPS2 was specifically required for LPS-induced Abca1 expression and cholesterol efflux, independent of LXRα. In addition, we identified a functional cooperation between the NF-κB subunit p65 and GPS2 based on ChIP assays, as the knockdown of either of them decreased the chromatin recruitment of the other at the *Abca1* locus. Further, the double knockdown of p65 and GPS2 eliminated LPS-induced *Abca1* expression. Finally, loss of GPS2 abrogated LXRα trans-repression of pro-inflammatory gene expression (e.g., *Ccl7*), although it had minor effects on LXRα-induced *Abca1* expression. Overall, this work identifies a regulatory chromatin component of crosstalk mechanisms between cholesterol efflux and inflammation that affects ABCA1 specifically (Figure 9).

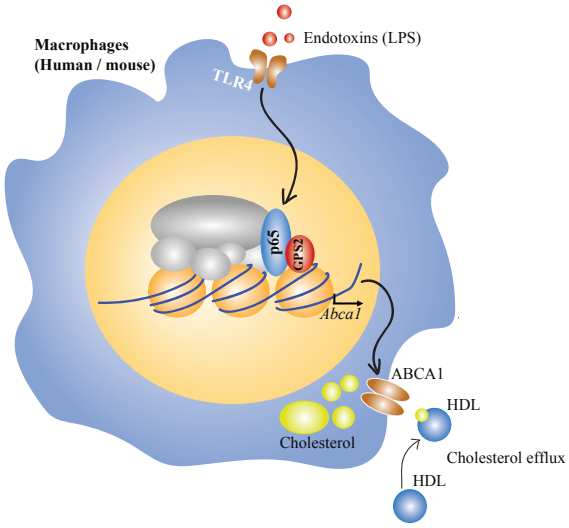

Figure 9 Model illustrating the cooperation of GPS2 and p65 in mediating LPS-induced cholesterol efflux in macrophages

Reprinted from "Huang, Z., et al. (2019). G protein pathway suppressor 2 (GPS2) links inflammation and cholesterol efflux by controlling lipopolysaccharide-induced ATP-binding cassette transporter A1 expression in macrophages. FASEB J 33(2): 1631-1643. doi: 10.1096/fj.201801123R" under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

4.3 PAPER III: Impaired LXRα phosphorylation attenuates progression of fatty liver disease

PTMs represent major mechanisms to modify NR activity and function. Despite the well-recognized importance of LXRα in maintaining hepatic metabolic homeostasis, it is unclarified how the PTMs affect LXRα function and influence liver metabolic diseases. To directly address the impact of LXRα phosphorylation on NAFLD progression, we generated a mouse model harboring an S196A mutation that disrupts LXRα phosphorylation at Ser196. Upon high-fat high-cholesterol diet (HFHCD), S196A mice displayed enhanced steatosis, but a significantly attenuated progression to steatohepatitis. The gene expression analysis showed a unique diet-induced transcriptome that prevents cholesterol accumulation and reduces hepatic inflammation and fibrosis. Furthermore, ChIP(-seq)s of LXRα, TBLR1 and H3K27ac were performed to understand the mechanism underlying the gene regulation. We found that phospho-deficient LXRα altered LXRα and TBLR1 cofactor occupancy and promoted H3K27 acetylation at regulatory sites for pro-fibrotic and pro-inflammatory genes. Overall, impaired LXRα-Ser196 phosphorylation acts as a novel nutritional molecular sensor that profoundly alters the hepatic H3K27ac and transcriptome during NAFLD progression (Figure 10).

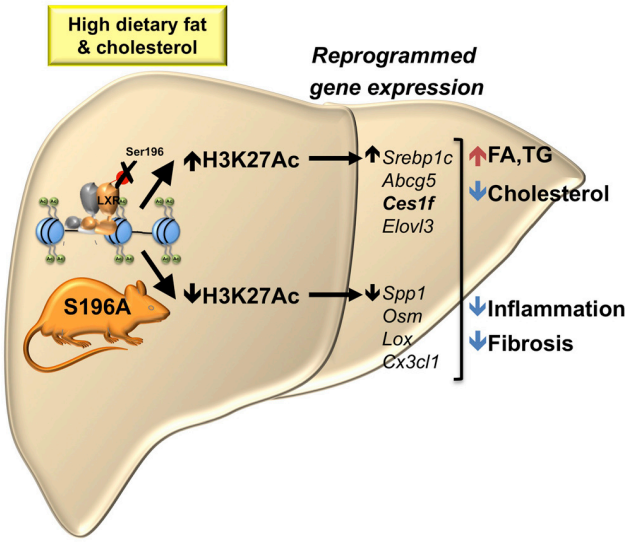

Figure 10 Model illustrating that phospho-deficient LXRα promotes chromatin modifications and regulates hepatocyte response to the HFHC diet in a gene-specific manner.

5 DISCUSSION

In study I, we discovered that GPS2 triggers the progression of NAFLD/NASH by repressing PPARα in mice, and probably in humans. PPARα de-repression in LKO mice alleviated liver steatosis upon HFD feeding and improved fibrosis upon MCD feeding, due to increased lipid burning as detected by elevated ketone body levels. To be noted, the protective phenotype of the *Gps2* LKO mice is unique amongst hitherto described coregulator LKO mouse models in the context of NAFLD as it is the only model which improved diet-induced NAFLD instead of worsening it. This study also provides a unique resource of hepatocyte ChIP-seq data as we determined chromatin occupancy for GPS2, NCOR, and PPARα, along with H3K27ac enhancer mark, in WT and the respective KO livers depleting each of these factors. Furthermore, this hepatic function of GPS2 appears to be conserved between mice and humans as GPS2 mRNA levels correlated with fibrogenic and inflammatory gene expression in human NAFLD/NASH livers. This study might provide hepatocyte-based epigenomic explanations for the diverse susceptibility in NAFLD/NASH patients to develop more severe stages of liver fibrosis and ultimately liver cancer, in addition to alterations in other cell types such as liver-resident immune cells (5).

In study II, we demonstrated that GPS2 has a unique function in governing endotoxin (LPS)-induced macrophage cholesterol efflux via ABCA1, through a regulatory mechanism that is conserved in mouse and human macrophages. We have reported that the SMRT/GPS2 corepressor sub-complex crucially controls the macrophage epigenome during activation by metabolic stress (90). Complementary to that work, study II improves our understanding of GPS2 functions in another aspect, for example, how a corepressor complex subunit GPS2 regulates macrophages signaling pathways that link obesity-associated inflammation and cholesterol efflux to the development of cardiovascular diseases. In particular, our results contribute to re-defining the function of GPS2 and ABCA1 in anti-inflammatory trans-repression mechanisms (82, 114, 168). Although GPS2 protein itself is currently unlikely to be a feasible target for drug development, the GPS2-ABCA1-LXR axis may provide a potential therapeutic target for further investigation of anti-inflammatory drug candidates.

In study III, we reported that disrupting phosphorylation at Ser196 (S196A) in LXRα retarded NAFLD progression upon HFHCD by repressing the pro-inflammatory and pro-fibrotic mediators, although it enhanced liver steatosis due to the activation of DNL. Besides, the integrated analysis of the transcriptome and H3K27ac uncovered a class of diet-specific and phosphorylation-sensitive genes, which are not regulated by synthetic LXRα ligands.

Thus, the LXRα-S196A mutation regulates hepatic transcription in a gene-dependent manner, rather than conferring an overall gain or loss of LXRα function. In addition, the phosphorylation-sensitive *Ces1* gene cluster has been reported to be protective from liver inflammation and injury (169, 170), although its specific contribution to NAFLD needs to be further addressed. Overall, our findings identify LXRα phosphorylation as an anti-inflammatory and anti-fibrotic therapeutic target, alternatively to synthetic ligands.

6 CONCLUDING REMARKS AND PERSPECTIVES

The three studies included in this thesis focused on the roles of two NRs (PPARα and LXRα) and the corepressor complex subunit GPS2 in obesity/T2D-related alterations of lipid and cholesterol metabolism. This thesis applied both *in vivo* and *in vitro* models to investigate the cell-type-specific functions of these NRs and GPS2 in hepatocytes and macrophages respectively. By doing so, this thesis revealed novel insights into mechanisms underlying NAFLD and atherosclerosis. Therefore, this thesis may contribute to our deeper understanding of not only the development, but perhaps also the prevention and future therapeutic treatment of these diseases.

Hepatocytes are the main cell type in the liver responsible for lipid metabolism (28). The maintenance of metabolic homeostasis is mainly coordinated at the level of gene expression via transcriptional networks composed of TFs, in particular NRs, and associated coregulators, including chromatin-modifying complexes. Disturbance of these transcriptional networks can lead to dysregulated lipid and glucose metabolism and has been linked to the progression of NAFLD (5, 6).

Utilizing *Gps2* LKO mice, we found that depletion of hepatic GPS2 releases the inhibition of PPARα targets by the corepressor complex. These PPARα targets include not only the local FAO enzymes to aid the hepatic lipid burning, but also endocrine hormone FGF21 to regulate glucose and lipid homeostasis and insulin sensitivity in pleiotropic tissues (49, 171, 172). This provides new evidence for metabolic organ crosstalk, e.g., how signaling pathways in the liver could affect the metabolism in other tissues.

It has been previously reported that a SMRT/GPS2 corepressor sub-complex controls pro-inflammatory gene activation upon obesity-linked metabolic stress in macrophages (86, 90). Intriguingly, mechanistic results of the our *Gps2* LKO study suggests that the metabolic function of GPS2 in the liver depends on NCOR but not on SMRT. Currently, the reasons for this cell type-selective cooperativity are still unclear, but the function of GPS2 within different sub-complexes represents a hitherto unrecognized feature.

Paper I is also amongst the very few corepressor-focused studies that have integrated human data and compared mouse and human liver pathways both at the physiological and (epi)genomic levels. Despite the high evolutionary conservation of corepressors and their

complexes, their genomic targets, i.e. the regulatory promoters and enhancers, can be highly divergent between humans and mice.

Besides the *in vivo* studies using hepatocyte or macrophage *Gps2* KO mice, we also utilized GPS2-depleted macrophage cell lines and are currently establishing related hepatocyte cell lines. The *in vitro* studies, on one hand, enabled us to verify and test potential mechanisms underlying the phenotypes of the *Gps2* KO mice. On the other hand, they also resulted in new complementary findings that would have been difficult to detect *in vivo*. For example, we found that GPS2 in macrophages also functions as a p65 co-activator independently of the complex, indicating that there is a fraction of 'free' GPS2 involved in repression-independent transcriptional regulation. Whether GPS2 has related function in hepatocytes remains to be studied.

PTMs such as phosphorylation could provide an essential mechanism by which NRs or coregulators can switch targets and selectively modulate liver metabolism (98, 148). Using LXRα-S196A knockin mice, we showed the physiological consequences of disrupting LXRα phosphorylation on NAFLD progression. This may provide an alternative therapeutic target for NAFLD aiming the PTMs of LXRα. More generally, we should study much more the signal-regulated PTMs that potentially play a major role in the target selection and the corepressor/coactivator switch. These issues are highly relevant for the better understanding and future targeting of liver disease pathways triggered by aging, nutrition and life style, such as obesity-associated NAFLD and hepatic insulin resistance. Finally, regarding therapeutic strategies, pathway-specific intervention using rapidly evolving RNA/protein-targeting technologies may be possible in near future, but research efforts utilizing humanized liver disease models should before scrutinize the pros and cons of targeting hepatic TF-corepressor networks.

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